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Changes in the micro- and nanostructure of siliceous valves in the diatom *Synedra acus* under the effect of colchicine treatment at different stages of the cell cycle

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ABSTRACT

The important role of the cytoskeleton in the morphogenesis of siliceous frustule components, which are synthesized within the diatom cells, has been revealed due to experiments with microtubule inhibitors. It has been shown that colchicine entering the diatom cell inhibits polymerization of tubulin, the main protein of microtubules, thereby disrupting the normal processes of biogenic silica deposition and daughter valve morphogenesis. In this study, experiments with a synchronized culture of the pennate diatom *Synedra acus* have been performed to determine the timing and duration of the formation of various valve components and analyze the effect of colchicine at a subtoxic concentration on the structure of daughter valves at different stages of their morphogenesis. Electron microscopic analysis has revealed several types of micro- and nanoscale anomalies in daughter valve morphology, with their frequency varying depending on the time of colchicine treatment. Laser scanning microscopy of preparations vitally stained with Tubulin Tracker Green has shown that polymerized tubulin at early stages of valve morphogenesis is localized along the periphery of the developing valve. This is evidence for an important role of microtubules in the horizontal growth of the valve at the stage when its general structural pattern is established, including its shape and arrangement of basic micro- and nanostructures. Treatment with a microtubule inhibitor at a certain stage of valve morphogenesis makes it possible to obtain new forms with a specific structure of siliceous components that hold promise for use in nanotechnologies.

1. Introduction

Diatoms are unicellular, mostly autotrophic organisms covered with a protective shell (frustule) of amorphous silica that consists of two valves with various surface structures (slits, ridges, processes, pores, etc.) arranged in a specific pattern (Round et al., 1990). Diatom frustules widely differ in this pattern as well as in symmetry, shape, and macro- and microstructure. It is noteworthy that they have found various biotechnological applications, e.g., as microcapsules for drug delivery (Gordon et al., 2012), sorbents with specified pore sizes, and filters and biosensors with unique properties (Dolatabadi and Guardia, 2011).

It is known that siliceous components of the frustule are synthesized within the cell, in a special membrane-bound compartment named silica deposition vesicle (SDV) (Reimann, 1964; Drum and Pankratz, 1964), but the mechanisms controlling the process of silica deposition and valve morphogenesis have not yet been studied sufficiently (Kröger and Poulsen, 2008).

The cytoskeleton of diatoms not only plays a major role in cell division, as in other eukaryotes, but is also involved in valve morphogenesis, and inhibition of polymerization of tubulin, the main microtubule protein, leads to various disturbances in this process. In the marine centric diatom *Coscinodiscus granii* Gough, confocal microscopic analysis has revealed a branched microtubule network radiating from the central portion of a cell undergoing new valve synthesis and closely associated with the proximal surface of the newly forming valve, determining its position relative to the mother cell valve (Tesson and Hildebrand, 2010a).

Transmission electron microscopy (TEM) of ultrathin sections of the marine bicentric diatom *Chaetoceros decipiens* Cleve has shown that microtubules form a supporting "sleeve" in siliceous setae of the new valve (Pickett-Heaps, 1998). During valve morphogenesis in the marine centric diatom *Proboscia alata* (Brightwell) Sundström, a similar sleeve of bundled microtubules is formed in the growing proboscis tip of the new valve. When its morphogenesis is completed, microtubules are distributed over the

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cytoplasm. Treatment of living *P. alata* cells with the microtubule inhibitor oryzalin (0.5 μM) results in overall distortion of proboscis tips (Van de Meene and Pickett-Heaps, 2002). According to TEM data, microtubules show tight packing along the raphe and determine its location and shape during valve morphogenesis in *Navicula cuspidata* (Kützing) Kützing, and the same is true of the raphe canal in *Surrirela robusta* Ehrenberg and *Hantzschia amphioxys* (Ehrenberg) Grunow (Pickett-Heaps et al., 1990). Inhibition of microtubule polymerization with 5 μM colchicine in the marine raphid pennate diatom *Pinnularia* sp. Ehrenberg resulted in lateral displacement of the raphe (Cohn et al., 1989). The effect of colchicine on valve morphogenesis in the canal raphid pennate diatoms *H. amphioxys* and *S. robusta* is manifested in fusion of the raphe canal with the valve surface in the former and disturbances in the formation of valvar wings located around the valve face in the latter (Cohn et al., 1989). It has been shown that microtubules participate in positioning of submicrometer structures such as rimoportulae in *C. granii* (Tesson and Hildebrand, 2010a) and that inhibitors of microtubule polymerization (colchicine and oryzalin) alter the positions of fultoportulae (about 300 nm in size) and the structure of the valve edge at the submicrometer scale in *Cyclotella cryptica* Reimann, Lewin & Guillard (Tesson and Hildebrand, 2010b).

Although detailed data are available on valve morphogenesis in *Gephyria media* W. Arnott (Tiffany, 2002), *Synedra acus* subsp. *radians* (Kütz.) Skabitsch. (Kaluzhnaya and Likhoshway, 2007), *Rhaphoneis amphiceros* (Ehr.) Ehr. (Sato et al., 2011), and *G. media* Arnott (Tiffany, 2002), the role of microtubules in the formation of certain structures in the siliceous valves of araphid pennate diatoms has not been investigated. The object of this study is the freshwater araphid pennate diatom *S. acus* cultivated under laboratory conditions (Safonova et al., 2007a). This diatom has long been used as a model for analyzing mechanisms of silicic acid transport into the cell and deposition of biogenic silica (Grachev et al., 2002, 2008; Sherbakova et al., 2005; Annenkov et al., 2013) and for genomic (Ravin et al., 2010; Galachyants et al., 2011) and molecular biological research (Gabaev et al., 2008; Khabudaev et al., 2014). Data have been obtained on the fine structure of *S. acus* cells (Bedoshvili et al., 2007, 2009, 2012), early steps in the formation of siliceous valves and consecutive stages of their morphogenesis (Kaluzhnaya and Likhoshway, 2007), and the effect of germanium on this process (Safonova et al., 2007b).

The purpose of this study was to analyze in detail the role of microtubules at different stages of *S. acus* valve morphogenesis using several microscopic methods and inhibition of microtubule polymerization with colchicine. To exclude the inhibitory effect of colchicine on mitosis, experiments were performed with cultures synchronized by silicon starvation, which resulted in cell cycle arrest after mitosis and cytokinesis, before the onset of valve formation. This approach allowed us to reveal the distribution pattern of polymerized tubulin, estimate the duration of basic stages of siliceous valve morphogenesis, and evaluate the effect of colchicine treatment at certain stages of the cell cycle on the fine structure of the valves.

2. Material and methods

2.1. Culture conditions

The diatom *S. acus* subsp. *radians* from a natural Lake Baikal population was cultured in DM medium (Thompson et al., 1988) at 7–8 °C and a 12-h photoperiod, with illumination intensity being 13–16 μmol/m^2 s.

To synchronize the culture, diatom cells were pelleted in a 5415R centrifuge (Eppendorf, Germany) at 1000*g* for 10 min at 10 °C, resuspended in 50 mL of silicon-free DM medium, inoculated in 200-mL plastic culture flasks (TPP, Switzerland), and incubated at 10 °C in the dark. After 24 h, a control cell sample (zero time point) was taken, and the culture was supplemented with sodium metasilicate ($Na_2SiO_3 \bullet 9H_2O$) to a final concentration of 57 μg/mL and incubated at 7–8 °C under illumination (13–16 μmol/m^2 s).

2.2. Determination of subtoxic concentration of colchicine

Diatom cells from nonsynchronized culture were plated in a 96-well CELLSTAR culture plate (well working volume 200 μL) and incubated with different concentrations of colchicine (1, 0.5, 0.1, 0.05, 0.01, and 0.001 μg/mL) or without it (control) for 4 days. The plate was periodically examined under an Axiovert 200 microscope (Zeiss, Germany) to count all cells in each well. The results were used to plot cell growth curves, which were compared to determine the required concentration of colchicine. The experiment was performed in triplicate. The measurement error in this and other experiments was estimated using the Statistica 6 program.

2.3. Colchicine treatment at different stages of valve morphogenesis

Colchicine (final concentration 0.5 μg/mL) was added to synchronized *S. acus* culture in 200-mL flasks at intervals of 0.5, 1.5, 2.5, and 3.5 h after the culture was replenished with silicon (see above). After 0.5-h exposure, diatom cells were washed with distilled water, pelleted at 1000*g* for 10 min, transferred to fresh DM medium with silicon, incubated for 24 h, pelleted again, and fixed with 70% ethyl alcohol. The experiment was performed in triplicate.

2.4. Laser scanning microscopy (LSM)

Microtubules were labeled with Tubulin Tracker Green (Oregon Green 488 Taxol, bis-acetate) (Life Technologies, United States), which specifically stains polymerized tubulin in live cells. TubulinTracker™ Green reagent was diluted in 71 μL of anhydrous DMSO. Staining was performed according to the manufacturer's protocol: diatom cells in DM medium were treated with 250 nM Tubulin Tracker Green for 30 min at 37 °C. Thereafter, the cells were washed in two portions of DM medium, pelleted at 1000*g* for 10 min, and examined under an LSM 710 laser scanning microscope with a Plan-Apochromat 63×/1.40 Oil DIC M27 objective lens (Carl Zeiss, Germany). The fluorescence excitation and emission wavelength for Tubulin Tracker were 488 and 496–647 nm; those for spontaneous chloroplast fluorescence, 561 and 650–723 nm, respectively. Microscopic images were processed using the ZEN 2010 program (Carl Zeiss).

2.5. Preparation of frustule valves for electron microscopy

Diatom cells were boiled in three changes of 6% SDS solution (UngerFabrikker AS, Norway) in a water bath for 30 min each, with intermediate washing in five portions of distilled water, and collected in a 1.5-mL tube (Axygen, United States). Thereafter, they were treated with concentrated nitric acid (OOO Reaktiv, Russia) in a water bath for 1 h, washed in three portions of ethyl alcohol, treated with concentrated hydrochloric acid for 24 h, and washed in at least five portions of distilled water. After each step, the material was pelleted by centrifugation at 1000*g* for 10 min.

2.6. Transmission electron microscopy (TEM)

The suspension of cleaned valves (50 μL) was pipetted onto a formvar-coated copper grid and examined in a Leo 906 E microscope (Carl Zeiss) at an acceleration voltage of 80 kV. Microscopic images were made with a MegaView II camera (Carl Zeiss). In each